PATENT
Attorney Docket No. 015119-000430US

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re the application of:

Stephen Withers *et al.*

Application No.: 09/837,711

Filed: April 17, 2001

For: Methods and Compositions for the Synthesis of Oligosaccharides Using Mutant Glycosidase Enzymes

Examiner: E. Slobodyansky

Art Unit: 1652

DECLARATION UNDER 37 C.F.R. § 1.132

Assistant Commissioner for Patents
P.O. Box 1450
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Dear Sir:

1. The undersigned, a named inventor of the above-identified Application, hereby submits the following declaration in support of the patentability of the invention disclosed and claimed in the Application.

2. This declaration describes experiments demonstrating the use of alternative glycosyl donors other than glycosyl fluorides in transglycosylation reactions catalyzed by a mutant glycosidase of the present invention. The experiments have been carried out under my direction and control and are part of ongoing studies into the characteristics of the mutant enzymes of the invention. The following summarizes experiments using glycosyl formates and glycosyl azides as alternative donors to glycosyl fluorides with a mutant β-glycosidase. The glycosyl donors used in the experiments are not specifically listed as examples in the specification, but would be considered in the art by a skilled artisan to be glycosyl donors that

are modified by a group that is "reasonably small and which function as relatively good leaving group[s]" as defined in the specification at page 12, lines 3 through 11.

3. In the experiments described the alternative glycosyl donors were synthesized *in situ* by the enzyme itself; however, these donors can be independently synthesized by chemical means and employed directly. Rescue reactions were performed in which an external nucleophile of the appropriate size (azide, formate, *etc.*) was combined with an activated glycoside with the anomeric stereochemistry of the natural substrate along with the mutant glycosidase of the present invention and a glycoside acceptor. The external nucleophile takes the place of the nucleophile removed from the active site of the mutated enzyme, with the added nucleophile replacing the DNP of the donor with inversion of anomeric configuration, thereby forming the modified glycosyl donor. These two steps, formation of the donor sugar and coupling, were carried out in a single reaction vessel, but are described separately below for clarity:

a) In the first (rescue) step the modified glycosyl donors α-glucopyranosyl fluoride, α-glucopyranosyl formate and α-glucopyranosyl azide were formed. See Figure 1A. In particular, the modified glycoside dinitrophenyl-β-D-glucopyranoside (12 mM) was incubated in the presence of about 100 mM fluoride (KF), formate ($NaHCO_2$) or azide (NaN_3) with the nucleophile mutant *Agrobacterium* E358G β-glucosidase (Abg E358G; about 2.5 mg/ml) in phosphate buffer (60 mM) for 3 hours at room temperature.

b) In the second (coupling) step glycosynthase reaction each modified glycosyl donor produced in the first step was transglycosylated onto the acceptor *p*NP β-D-glucopyranoside. See Figure 1B. In particular, α-glucopyranosyl fluoride, α-glucopyranosyl formate or α-glucopyranosyl azide formed in each of the first rescue reactions was incubated in the presence of the glycoside acceptor *p*NP β-D-glucopyranoside (12 mM) with the nucleophile mutant Abg E358G (about 2.5 mg/ml) in phosphate buffer (60 mM) at room temperature for 3 hours.

4. With all three putative external nucleophiles (F^-, HCO_2^-, and N_3^-) formation of the glycosyl donor was indeed observed as indicated by an increased rate of dinitrophenol (DNP) release compared to that in a control reaction containing no external nucleophile. Thin layer chromatography (TLC) experiments demonstrated that with all three newly formed glycosyl donors a transglycosylation (coupling) reactions occurred as demonstrated by the formation of new UV-active compounds. ESI/MS experiments showed that indeed transglycosylation had occurred, as could be seen by newly formed signals corresponding to the m/z of a *p*NP disaccharide (m/z = 486.5) and a *p*NP trisaccharide (m/z = 648.5) in the case of azide and fluoride and to the m/z of a *p*NP disaccharide, a *p*NP trisaccharide and a *p*NP tetrasaccharide (m/z = 810.7) in the case of formate (all as $[M+Na]^+$).

5. From these experiments I conclude that modified glycosyl donors as defined in the specification as filed, *e.g.*, glycosyl formates and glycosyl azides, can serve as modified glycosyl donor molecules in the same manner as the glycosyl fluorides specifically exemplified in the present application when used with a mutant glycosidase of the present invention.

I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that I make these statements with the knowledge that willful false statements and the like are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize validity of the application or any patent issuing thereon.

Date: 19th November 2003 By: SGW
Stephen G. Withers